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Dendritic Cell-Mediated Induction of Mucosal Cytotoxic Responses following Intravaginal Immunization with the Nontoxic B Subunit of Cholera Toxin

Carmelo Luci,* Catherine Hervouet,* Déborah Rousseau,* Jan Holmgren,† Cecil Czerkinsky,* and Fabienne Anjüere2*

The use of the nontoxic B subunit of cholera toxin (CTB) as mucosal adjuvant and carrier-delivery system for inducing secretory Ab responses has been documented previously with different soluble Ags. In this study, we have evaluated this approach for inducing CTL responses against a prototype Ag, OVA, in the female genital mucosa. We report here the ability of an immunogen comprised of CTB conjugated to OVA (CTB-OVA) given by intravaginal (ivag) route to induce genital OVA-specific CTLs in mice. Using adoptive transfer models, we demonstrate that ivag application of CTB-OVA activates OVA-specific IFN-γ-producing CD4 and CD8 T cells in draining lymph nodes (DLN). Moreover, ivag CTB induces an expansion of IFN-γ-secreting CD8+ T cells in DLN and genital mucosa and promotes Ab responses to OVA. In contrast, ivag administration of OVA alone or coadministered with CTB failed to induce such responses. Importantly, we demonstrate that ivag CTB-OVA generates OVA-specific CTLs in DLN and the genital mucosa. Furthermore, genital CD11b+CD11c+ dendritic cells (DCs), but not CD8+CD11c+ or CD11c+ APCs, present MHC class I epitopes acquired after ivag CTB-OVA, suggesting a critical role of this DC subset in the priming of genital CTLs. Inhibition studies indicate that the presentation of OVA MHC class I epitopes by DCs conditioned with CTB-OVA involves a proteasome-dependent and chloroquine-sensitive mechanism. These results demonstrate that CTB is an efficient adjuvant-delivery system for DC-mediated induction of genital CTL responses and may have implications for the design of vaccines against sexually transmitted infections. The Journal of Immunology, 2006, 176: 2749–2757.

Mucosal surfaces and vaginal mucosa in particular are under constant exposure to infectious pathogens. The cervicovaginal mucosa consists in a highly specialized immune system, including effector mechanisms, such as secretory Ab production and T cell responses, playing a crucial role in the protection against pathogen invasion in both humans and rodents (1, 2). The most effective immune protection observed against a classical infection of the female genital tract (HSV-2 infection) was achieved by prior local immunization with an attenuated form of the virus and the induction of both secretory Ab production and cellular responses including cytotoxic T cell responses (3).

Following mucosal administration, soluble proteins are poorly immunogenic and favor a state of immunological unresponsiveness known as “peripheral tolerance” avoiding undesired inflammatory responses toward innocuous Ags. Therefore, to be effective against mucosal infections, vaccines have to stimulate both humoral and cellular immunity without inducing tolerance. The use of mucosal adjuvants appears to be one of the most promising approaches for the development of mucosal antiinfectious vaccines.

Cholera toxin (CT)3 constitutes one of the most powerful non-replicative mucosal adjuvants together with the heat-labile entero-toxin from Escherichia coli (LT) (4, 5). CT is able to induce local and systemic immunity against coadministered Ags when given at the same time by the same mucosal route, but due to its toxicity, CT is not usable in clinical trials (6). CT has an AB5 structure composed of two distinct subunits: a single toxic A subunit and a nontoxic homopentamer B subunit (nontoxic B subunit of CT (CTB)), with strong affinity for GM1 gangliosides present on all mammalian nucleated cells, which is responsible for the anchor of the toxin onto host cells (7). Interestingly, the use of the CTB as mucosal adjuvant and/or carrier-delivery system for inducing secretory Ab responses has been documented in several animal systems and in humans with a variety of soluble and particulate Ags (6).

The generation of mucosal-specific CTLs is described to play a crucial role in the early defense against tissue dissemination of various sexually transmitted infectious agents (such as HSV, human papillomavirus, and HIV...) (2, 8). Several studies have described the ability of native and mutant bacterial enterotoxins used as mucosal adjuvants to prime systemic or mucosal CTL responses. Bowen et al. (9) showed that oral coadministration of CT with OVA protein generates Ag-specific class I-restricted CTLs. Other studies have reported that CT and LT as well as a LT mutant with reduced toxicity were able to induce CTL responses against coadministered Ags by mucosal route (10–12). The induction of

3 Abbreviations used in this paper: CT, cholera toxin; CTB, B subunit of CT; LT, Escherichia coli heat-labile enterotoxin; ivag, intravaginal; DLN, draining lymph node; DC, dendritic cell; TC, tricolor; RT, room temperature; BM-DC, bone marrow-derived DC.
genital CTLs by local immunization with a nonreplicative mucosal adjuvant remains to be evaluated. Interestingly, the nontoxic CTB has been found to function as a useful carrier protein for induction of mucosal and systemic Ab responses against a colinked Ag when administered by genital route (13), suggesting it might constitute an adjuvant of genital T cytotoxic responses following local immunization.

An effective cellular immune response requires that APCs process Ag and present epitopes to T cells. Dendritic cells are known to be the most potent APCs able to prime both CD4+ and CD8+ T cells. DCs act as sentinels, playing a central role in immunity as they are located in most nonlymphoid tissues and in close contact with the mucosal surfaces where they can sample incoming pathogens (14). In the murine vaginal mucosa, DCs are present as Langhans cells in the epithelium layer (15). Furthermore, a subset of CD11c+CD11b+ DCs present in the vagina submucosal lamina propria are described to play a critical role in Th1 priming following vaginal HSV-2 infection (16).

The uptake of an exogenous soluble Ag by a professional APC leads to its intracellular association with MHC class II molecules and subsequent priming of CD4+ T cells. Exogenous Ags may also be presented in the MHC class I pathway by a mechanism called “cross-presentation” leading in certain conditions to the activation of cytotoxic CD8+ T cells (17). DCs are described to be the only APCs endowed with cross-presentation property as reviewed in Guermonprez et al. (18). Different adjuvant formulations are described to favor the induction of CD8+ T cells against exogenous Ags (19). Recent studies showed that the covariant coupling of nonreplicative Ags to bacterial B subunits of enterotoxins constituted efficient vector-delivery systems allowing the MHC class I-dependent presentation of peptides by DCs (20–22).

In that context, the aim of our study was to evaluate the potential of a subunit prototype vaccine comprised of CTB conjugated to OVA (CTB-OVA) to induce specific cytotoxic responses in the female genital tract of progesterone-treated mice after intravaginal (ivag) immunization. We demonstrated that ivag CTB-OVA leads to the priming of IFN-γ and IL-10-producing OVA-specific CD4 and IFN-γ-producing CD8 T cells. More interestingly, ivag CTB-OVA induced the in vivo expansion of OVA-specific CD8+ T cells in DLN and the genital mucosa itself. Importantly, we demonstrated that ivag CTB-OVA generates OVA-specific CTLs in both draining lymph nodes (DLN) and genital mucosa itself. Furthermore, genital CD11b+CD11c+ DCs were able to present MHC class I epitopes acquired after ivag CTB-OVA, suggesting a critical role of this specific CD11c+ DC subset in the priming of genital CTLs. Finally, inhibition studies indicated that DCs are able to present MHC class I-restricted OVA epitopes derived from exogenous CTB-OVA by a proteasome-dependent and chlороquine-sensitive mechanism. These results further demonstrated the adjuvant properties of CTB toward genital CTL responses by a mechanism involving local DCs.

Materials and Methods

Animals

Female BALB/c and C57BL/6 mice were purchased from Charles River Laboratories. In all experiments, 6- to 8-wk-old female mice were used. OVA-TCR-transgenic mice (D011.10) on a BALB/c background expressing the αβ-TCR specific for the OVA epitope (323–339) recognized in the context of the MHC II molecule I-Aα (23) and OVA-TCR-transgenic mice (OT-1) on a C57BL/6 background expressing the αβ-TCR specific for the OVA epitope (257–264) recognized in the context of the MHC I molecule Kα (24) were bred in the animal facilities of the Faculty of Medicine, (Nice, France). All mice were maintained in common mouse pathogen-free conditions according to institutional guidelines.

CTB induces mucosal cytotoxic responses

Purified CT was obtained from List Biological Laboratories. rCTB was produced in a mutant strain of Vibrio cholerae 01 deleted of its CT genes and transfected with a multicopy plasmid encoding CTB. The CTB used was purified from the culture medium by a combination of salt precipitation and chromatographic methods to eliminate bacterial contaminants. Albumin chicken egg (OVA) grade V was purchased from Sigma-Aldrich. OVA was conjugated to CTB or to itself using N-succinimidyl-3-(2-pyridyl-dithio-propionate) (Pharmacia) as described earlier (25). The resulting CTB-OVA conjugate retained both GM1-binding activity and CTB and OVA serological reactivity, as judged by a solid phase ELISA using GM1 as capture system and Abs to CTB and OVA as detection reagents (26).

The OVA23–39 (ISQAVHAAHAEINEAGR) and OVA273–286 (SIINFEKL) peptides were obtained from NeoMPS. LPS contamination of the different preparations was below 0.12 ng/ml as determined using the Limulus Amebocyte test Pyrogen Plus (Cambrex BioWhittaker).

Immunizations

Progesterone (Deproprovera)-treated mice anesthetized by isoflurane inhalation were immunized by vaginal route on days 0, 14, and 21 with a 20-μl saline solution containing CTB-OVA conjugate (40 μg) alone, admixed with CT (1 μg), or with GM1 gangliosides (5 μg; Sigma-Aldrich), CTB (20 μg) coadministered with OVA (20 μg), OVA-OVA conjugate (20 μg), OVA alone (20 μg), or CTB alone (20 μg). All the formulations contained the same amount of OVA. DLN (ileoociual and inguinal lymph nodes) were excised 7 days following the last immunization and cell suspensions were prepared for analysis. Alternatively, some mice were immunized by s.c. route with OVA or CTB-OVA using the same protocol.

Flow cytometry

Frequency of OVA-specific CD8+ T cells was analyzed after triple staining with FITC-conjugated anti-CD8a (clone 53-6-7; BD Biosciences), PE-labeled pro5 MHC Pentamer H-2Kb/SIINFEKL (Proimmune), and biotin-conjugated anti-CD62L (clone 53-6.7; BD Biosciences), and avidin-tylated Abs specific for B cells, macrophages, granulocytes, and DCs (clones RA3-6B2, F4/80, RB6-8C5, and N418, respectively) and avidin-labeled pro5 MHC Pentamer H-2Kb/SIINFEKL and CD8-Chyrome 5 (clone 53-6-7; BD Biosciences) for CD8 T cells. Phenotypic analysis of dendritic cells (DCs) was performed after triple staining with FITC-conjugated anti-CD11c (clone N418, homologate), PE-conjugated anti-CD8a (clone 53-6-7; BD Biosciences), and TC-conjugated anti-CD11b (clone M1/70; Caltag Laboratories). Biotin-conjugated rat IgG2a (clone B39-4; BD Biosciences), rat IgG2b (clone A95-1; BD Biosciences), and hamster IgGl (clone A19-3; BD Biosciences) were used as isotype controls. Before specific staining, cell surface FcRs were blocked by incubation with purified anti-FCγRII/III mAb (2.4G2 clone; BD Biosciences). All the staining steps were performed at 0°C in PBS containing EDTA and 3% FCS. Analysis was performed on a FACScan flow cytometer (BD Biosciences) at the Flow Cytometry Laboratory (IFR50; Faculty of Medicine, Nice, France) using CellQuest Pro software (BD Biosciences). Cell sorting was performed using a FACSVantage SE (BD Biosciences).

CFSE labeling of donor cells and adoptive transfer experiments

Peripheral (axillary, inguinal, popliteal, auricular, brachial, iliac) and mesenteric lymph nodes from D011.10 or OT-1 mice were collected. APCs from cell suspensions were depleted using a homemade mixture of biotinylated Abs specific for B cells, macrophages, granulocytes, and DCs (clones RA3-6B2, F480, RB6-8C5, and N418, respectively) and avidin-coated magnetic beads following manufacturer’s instructions (Dynabeads; Dynal Biotech). T cell purity was estimated >95% by FACS analysis. To directly follow transgenic T cells in recipient mice, they were labeled with CFSE (Molecular Probes). Briefly, transgenic T cells were washed in PBS solution supplemented with 3% FCS and 5 mM EDTA (PBS-EDTA-FCS), resuspended at 25 × 10^6/ml in PBS and incubated with CFSE at a concentration of 4 μM for 5 min at room temperature. CFSE-labeled T cells were washed twice with cold PBS and 8 × 10^6 cells were then resuspended in 200 μl of PBS and injected i.v. in recipient mice. Adoptively transferred CTLs were then received different formulations of the MHC class I-restricted epitope and following the in vivo priming, DLN (ileoociual and inguinal lymph nodes) were collected, and purified CD4+ and CD8+ T cells were used for subsequent proliferation and cytokine analyses as described below.

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**In vivo cytotoxic assay**

Analysis of in vivo cytotoxicity was conducted as described elsewhere (27). Briefly, syngeneic spleenocytes were divided into two populations and labeled with CFSE at a concentration of 4 μM (CFSE<sup>low</sup>) or 0.4 μM (CFSE<sup>high</sup>) as described above. CFSE<sup>low</sup> cells were pulsed with 1 μM Ova<sub>257–264</sub>, (SHINFEK) peptide for 1 h at 37°C in the dark, while CFSE<sup>high</sup> remained unpulsed. The two spleenocyte populations were then washed, mixed at equal numbers (15 × 10<sup>6</sup> total cells) and injected i.v. 6 days after the last immunization. Draining nodes and spleen from recipient mice were taken 15 h later to measure the in vivo cytotoxicity of target cells estimated by the loss of the CFSE<sup>high</sup>-Ag-pulsed population relative to the control CFSE<sup>low</sup>-Ag-pulsed population using flow cytometry. Alternatively, the detection of specific CTLs in the vagina mucosa were performed using a modified in vivo cytotoxic assay. Mice, anesthetized by isoflurane inhalation, received injections with equal numbers (4 × 10<sup>6</sup> total cells) of the two splenocytes populations (CFSE<sup>low</sup> pulsed vs CFSE<sup>high</sup> unpulsed) directly in the mucosa using an insulin syringe (BD Biosciences). Fifteen hours later, the vagina was excised and digested by dispase II (1.2 U/ml in RPMI 1640) (Roche Diagnostics) during 45 min at 37°C to recover cells for cytosis analysis.

**Cytokine assays**

Culture supernatants from in vitro OVA-stimulated CD<sup>4+</sup> or CD<sup>8+</sup> T lymphocytes purified from cell suspensions of DLN were assayed for IL-10 contents using ELISA kits (BioSource International) according to manufacturer’s instructions. Other cytokines analyses were conducted using sandwich ELISAs for IL-4 (coating Ab 1B11; homemade and biotinylated Ab BV6D-24G2; BD Biosciences), IL-5 (Ab TRFK5, homemade and Ab TRFK3; BD Biosciences), IFN-γ (Ab 4I-4A2 and Ab AN18) was analyzed using Ab pairs from BD Biosciences. Briefly, high-binding 96-well polystyrene plates (Nunc) were coated with 100 μl of either IL-4, IL-5, IFN-γ, and IL-10-coating Abs in 0.1 M phosphate buffer overnight at room temperature (RT) followed by a step of saturation of 60 min in PBS with 0.5% BSA (Sigma-Aldrich) for IL-4, IL-5, and IL-10 or in 1% nonfat milk powder with 0.12% Triton X-100 for IFN-γ. All steps were performed at RT and followed by three washes with PBS containing 0.05% Tween 20 (PBST). Supernatants were incubated for 2 h at RT in PBS with 0.5% BSA for IL-4, IL-5, and IL-10 or in PBST with 1% nonfat milk for IFN-γ. After washing, the wells were exposed for 1 h with correspondingly biotinylated Abs and developed by stepwise addition of avidin HRP and 3,3’,5,5’ tetramethyl benzidine/H<sub>2</sub>O<sub>2</sub> (Kirkegaard & Perry Laboratories).

**Measurments of serum and mucosal Ab levels**

Serum and mucosal Abs against CTB and OVA were determined by means of solid phase ELISA. Briefly, high-binding 96-well polystyrene plates (Nunc) were coated with either CTB (1 μg in 100 μl) or OVA (10 μg in 100 μl) overnight at RT and blocked for 90 min with the assay buffer (1% phosphate buffer containing dry milk, 0.012% Triton X-100, 100 μg/ml phenol red). After three washes with 0.05% PBST, serial 3-fold dilutions of test sera in the assay buffer were incubated in coated wells for 2 h. After washing, the wells were incubated for 1 h with HRP-conjugated goat Abs to mouse IgG1, IgG2a, and IgG2b (Southern Biotechnology Associates) applied alone or as a mixture. Solid phase-bound HRP activity was monitored spectrophotometrically after addition of the enzyme substrate. The titer of a serum was defined as the reverse of the highest dilution yielding an absorbance value at least equal to 3-fold that of background. Mucosal Ab titers were tested in vaginal washes of immunized mice collected by pipetting consecutively six times 50 μl of PBS in and out of the vagina.

**Preparation of APCs**

DCs were purified from DLN adapting a protocol previously described (28, 29). Briefly, organs were digested with collagenase A (Boehringer-Mannheim) and DNase I (Boehringer-Mannheim) in RPMI 1640 medium supplemented with 5% FCS, filtered through a stainless-steel sieve, and cell suspensions were washed twice in PBS solution supplemented with 5% FCS and 5 mM EDTA (PBS-EDTA-FCS) containing 5 mg/ml DNase I. Unwanted T cells were removed magnetically after sequential incubation for 30 min at 4°C with anti-CD3 (clone KT3-1.1) and with anti-rat Ig coated magnetic beads (Dynabeads; Dynal Biotech) at a 5:1 bead-to-cell ratio according to manufacturer’s instructions. DCs were then enriched by positive selection using anti-CD11c magnetic beads and cell sorting with MACS separation columns (Miltenyi Biotec) according to manufacturer’s instructions and the remaining CD11c<sup>−</sup> fraction was used as the nonprofessional APC population. The CD11c<sup>+</sup> DC preparation obtained by this technique had a purity of >95%. In some experiments, CD<sup>11c</sup><sup>−</sup> and CD11b<sup>−</sup> DC subsets were obtained by FACS sorting of enriched CD11c<sup>+</sup> preparations stained with mAbs specific for CD11c, CD8, and CD11b as described in Flow cytometry with a purity >98%. Immature bone marrow-derived DCs (BM-DCs) were prepared as previously described (30).

**Inhibition studies**

Brefeldin A, lactacystin, epoxomicin, and chloroquine were purchased from Sigma-Aldrich and tested at various concentrations (brefeldin A at 10, 5, and 2 μg/ml, lactacystin at 20, 10, and 5 μM, epoxomicin at 10, 5, and 2 μM, and chloroquine at 100, 50, 20, and 5 μM). BM-DCs were incubated with drugs at different concentrations for 1 h at 37°C and then pulsed with CTB-OVA in the presence or absence of CT or OVA<sub>257–264</sub> peptide in the presence of the same drugs during 2 h. The cells were then washed with cold medium and cocultured with CFSE-labeled OT-1 transgenic T cells. Proliferation of the specific CD8 T cells were analyzed 72 h later by flow cytometry following CFSE dilution.

**Statistical analysis**

One-way ANOVA was used to compare experimental groups and was followed by pairwise multiple comparisons using a Bonferroni t test or a Student-Newman-Keuls test. A p value <0.05 was considered significant. All statistic calculations were computed with Sigma Stat software (SPSS).

**Results**

**Genital administration of CTB-OVA induces the specific proliferation of IFN-γ- and IL-10-producing CD4<sup>+</sup> T cells in the DLN**

We first evaluated the ability of a CTB chemical construction carrying the OVA protein (CTB-OVA) to stimulate in vivo proliferation of OVA-specific transgenic CD4<sup>+</sup> T cells when administered by genital route into adoptively transferred recipient mice. For this purpose, progesterone-treated recipient BALB/c mice were infused with CFSE-labeled transgenic TCR-OVA<sup>+</sup> T cells expressing a TCR specific for the immunodominant OVA CD4<sub>323–339</sub> epitope (OVA<sub>323–339</sub>). Twenty-four hours later, these mice were immunized with different formulations by genital route. DLN were collected 4 days later and the proliferation of specific CD4<sup>+</sup> TCR-OVA<sup>+</sup> cells was analyzed by flow cytometry following CFSE dilution. The CTB-OVA conjugate induced a strong proliferation of specific T cells in comparison to OVA alone or coadministered with CTB as indicated by the percentages of cells that have accomplished six divisions (32, 9, and 11%, respectively) (Fig. 1A). The OVA-OVA conjugate was comparable to OVA alone (data not shown). Interestingly, the addition of GM1 gangliosides to the CTB-OVA formulation suppressed the enhancing proliferative property of OVA conjugated CTB (14% in comparison with 32%) (Fig. 1C). Moreover, coadministration of CT to CTB-OVA increased by 2-fold the percentage of dividing cells (60 vs 32%). No proliferation of specific T cells was detected when mice received PBS or CTB alone.

We then evaluated the cytokines produced in secondary cultures to investigate the type(s) of Th subsets involved in these responses. DLN CD4<sup>+</sup> T cells from immunized mice were cocultured with syngeneic spleen cells pulsed with the Th OVA<sub>323–339</sub> peptide and 3 days later supernatants were assayed for Th1 and Th2 cytokine contents. As shown in Table I, cultures from mice immunized with CTB-OVA in the absence or presence of CT produced high levels of IFN-γ (510 and 1400 ng/ml, respectively), whereas control groups had no detectable levels of IFN-γ. Moreover, IL-10 production was also detected in these two groups (400 and 950 pg/ml, respectively), whereas IL-4 and IL-5 cytokine levels were below detection limit in all groups (data not shown).

These data showed that CTB administered by genital route stimulates proliferative responses against a heterologous Ag covalently linked to itself and promotes the preferential differentiation of naive CD4<sup>+</sup> T cells into IFN-γ- and IL-10-producing cells.
CTB induces mucosal cytotoxic responses

The ability of the CTB-OVA conjugate to stimulate the proliferation of specific CD8⁺ T cells following genital immunization was also evaluated. Progesterone-treated C57BL/6 recipient mice were adoptively transferred with CFSE-labeled CD8⁺ transgenic TCR-OVA⁺ T cells expressing a TCR specific for the immunodominant OVA CD8 epitope (OVA257–264). Recipient mice were then immunized by genital route with different formulations as described previously and the specific proliferation of CFSE-labeled CD8⁺ T cells was measured in DLN. As shown in Fig. 1B, the genital administration of CTB-OVA induced a higher proliferation rate of specific T cells than OVA alone or coadministered with CTB (26, 10, and 11%, respectively). As before, the genital administration of CTB-OVA together with GM1 gangliosides partly suppressed the proliferative activity of CD8⁺ T cells (Fig. 1C). Genital immunization with CTB-OVA in the absence or presence of CT induced similar proliferation of specific CD8⁺ T cells (26 vs 29%).

IFN-γ production by CD8⁺ T cells from adoptively transferred mice was evaluated in supernatants of secondary cultures obtained by in vitro restimulation of DLN-purified CD8⁺ T cells cocultured with syngeneic spleen cells pulsed with the OVA257–264 MHC class I peptide. T cells from mice immunized with CTB-OVA in the absence or presence of CT secreted high levels of IFN-γ (400 ± 60 and 110 ± 30 ng/ml, respectively), whereas no detectable levels of IFN-γ (<15 pg/ml) were detected in the other experimental groups.

The genital administration of CTB conjugated to OVA induced the activation of specific CD8⁺ T cells producing high levels of IFN-γ. This demonstrates that the OVA immunodominant CD8 epitope is efficiently presented to T cells following genital immunization with OVA-conjugated CTB and suggests that CTB may constitute a good adjuvant delivery system for cytotoxic responses by genital route.

Genital immunization with CTB-OVA induces Ab and cellular responses against OVA

We then investigated whether the genital administration of CTB-OVA was able to promote cellular CD8⁺ responses as well as Ab responses toward OVA into wild-type C57BL/6 mice. Groups of progesterone-treated C57BL/6 mice were immunized at days 0, 14, and 21. Seven days later, both the frequency and cytolytic activity of CD8⁺ T cells specific for the OVA257–264 MHC class I-dependent immunodominant epitope of OVA as well as the presence of anti-OVA IgG and IgA Abs in genital secretions and plasma were evaluated.

The presence of activated CD8⁺ T cells expressing the immunodominant epitope OVA257–264 was examined in cell suspensions of genital mucosa, DLN, and spleen by flow cytometry analysis using a fluorescent SIINFEKL-Kb MHC class I pentamer. As shown in Fig. 2A, cell suspensions of DLN obtained from mice immunized with CTB-OVA had enhanced percentages of TCR-OVA⁺ T cells in the activated CD62L⁺CD8⁺ T cell subset in comparison with groups that received OVA alone or coadministered with CTB (4, 0.47, and 2.46, respectively). Addition of CT did not modify the rate of specific CD8⁺ T cells in DLN (4.43%) (Fig. 2A) but increased their percentage in spleen in comparison with other experimental groups (3.8% for CTB-OVA + CT vs 0.75% for CTB-OVA) (Fig. 2B). Levels of IFN-γ were assessed in supernatants of secondary cultures from DLN T cells cocultured with OVA257–264-pulsed syngeneic spleen cells. IFN-γ production
was observed in groups immunized with CTB-OVA (80 pg/ml) or CTB-OVA + CT (290 pg/ml), whereas no detectable levels of IFN-γ were detected in the other experimental groups. Interestingly, activated TCR-OVA+CD8+ T cells were detected in genital cell suspensions following immunization with CTB-OVA and CTB coadministered with CTB (7 vs 3.2%) indicating that specific effector T cells had migrated to the immunization site (Fig. 2C). To note that the frequency of effector T cells in the genital mucosa of mice immunized in the presence of CT has not been determined due to a lack of viability of extracted cells. Parallel s.c. immunization with CTB-OVA generated some TCR-OVA+ T cells (1.8%) in the genital lymph nodes but no specific T cells in the mucosa itself while s.c. OVA was not able to induce any specific T cell response (data not shown).

All animals immunized either with CTB alone or with OVA or CTB-OVA in the presence or absence of CT exhibited high plasmatic and genital anti-CTB IgG and anti-CTB IgA Ab titers (data not shown). Furthermore, in accordance with data obtained by Johansson et al. (13), the genital immunization with CTB-OVA induced notable plasmatic anti-OVA IgG Ab responses in all animals (mean titer 2100 ± 400; range 150–4,050; n = 10) in comparison with mice immunized with OVA coadministered with CTB (mean titer 300 ± 90; range 150–450; n = 10) demonstrating the carrier properties of CTB toward anti-OVA Ab responses. The addition of CT to CTB-OVA induced a 4-fold increase in anti-OVA IgG responses (mean titer 9,100 ± 1,500; range 4,050–12,125; n = 8). As shown in Table II, subclass analysis showed the presence of both anti-OVA IgG1 and IgG2a Abs at different levels in groups immunized in the presence of CTB in accordance with the mixed Th response observed above. In the same sense, low but consistent levels of anti-OVA IgA and IgG (mean titer 5 ± 2 and 9 ± 3, respectively; n = 4) were present in vaginal secretions of mice immunized with CTB-OVA, whereas no anti-OVA Ab were detected in groups which received OVA and OVA coadministered with CTB. Addition of CT potentiated the genital anti-OVA IgA and IgG responses by 2- to 3-fold (data not shown).

Altogether, these results demonstrate that CTB administered by genital route constitute an interesting adjuvant-delivery system for the induction of regional cellular CD8+ responses to a colinked heterologous Ag, a property which is complementary to the already known adjuvant activities of CTB toward Ab responses against a colinked Ag. The production of IFN-γ by these CD8+ T cells strongly suggests that CTB might be an adjuvant of cytotoxic responses.

**Genital immunization with CTB-OVA induces regional cytolytic CD8+ T cell responses**

We then evaluated the cytolytic potential of the specific CD8 T cells generated in vivo. To detect potential OVA-specific CTLs into immunized mice, we used an in vivo CTL assay consisting in i.v. infusion of OVA-loaded CFSE+ target cells followed by the subsequent ex vivo quantification of remaining OVA-loaded CFSE+ target cells in DLN and distant lymph nodes as well as in spleen cell suspensions by flow cytometry analysis. As shown in Fig. 3A, a partial lysis of CFSE+ OVA-loaded target cells was observed in mice immunized with CTB-OVA (corresponding to 30% of specific cytolytic), whereas immunization with OVA alone or coadministered with CTB was inefficient to induce anti-OVA CTLs. This cytolytic activity was mainly restricted to DLN as no lysis (Fig. 3B) observed in spleen. The addition of GM1 gangliosides to the CTB-OVA formulation impeded the generation of specific CTLs (Fig. 3). To note that even though genital immunization with OVA coadministered with CTB induced notable amounts of OVA-specific CD8+ T cells in DLN in comparison with the group which received OVA-conjugated CTB (2.46 vs 4%; Fig. 2A), these specific CD8+ T cells were devoid of cytolytic potential (Fig. 3). The addition of CT to OVA-conjugated CTB increased the lytic

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**Table II. Serum anti-OVA IgG isotype responses following ivag immunization**

<table>
<thead>
<tr>
<th>OVA-Specific IgG Subclasses</th>
<th>IgG1</th>
<th>IgG2a</th>
<th>IgG1/IgG2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 CTB + OVA</td>
<td>0.36 ± 0.16</td>
<td>0.018 ± 0.002</td>
<td>20</td>
</tr>
<tr>
<td>2 CTB - OVA</td>
<td>1 ± 0.25</td>
<td>0.1 ± 0.035</td>
<td>10</td>
</tr>
<tr>
<td>3 CTB - OVA + CT</td>
<td>1.26 ± 0.28</td>
<td>0.82 ± 0.17</td>
<td>1.5</td>
</tr>
</tbody>
</table>

*Groups of mice (n = 10) received three consecutive ivag immunizations at days 0, 14, and 21 with different formulations. One week later, diluted sera (1/50) were assayed by ELISA for anti-OVA IgG1 and IgG2a Abs. Values represent mean absorbance ± SEM.

*Groups 2 and 3 are significantly different from group 1 (p < 0.05, Student-Newman-Keuls test).
The contribution of DLN CD11c+ DCs in T cell priming after genital immunization with CTB-OVA was then evaluated. Highly enriched CD11c+ DCs and CD11c- APCs were prepared from DLN 36 h following immunization either with OVA-conjugated CTB or OVA alone and were cocultured with CFSE-labeled purified transgenic T cells from DO11.10 or OT-1 transgenic mice. CD11c+ DCs isolated from mice immunized with CTB-OVA were able to present ex vivo OVA CD4 and CD8 peptides to the specific transgenic T cells and to induce their proliferation as shown by the increase of the dividing cell population (50% for CD4 responses and 80% for CD8 responses) (Fig. 4, A and B, respectively), whereas the genital administration of OVA alone had limited effect on genital DC Ag-presenting capability. The addition of CT together with CTB-OVA did not improve the presenting ability of CD11c+ DCs, suggesting that CT had no maturation effect on DLN DCs following genital administration (Fig. 4). Genital DCs isolated from mice immunized with CTB-OVA 6 h before analysis instead of 36 h were unable to induce the proliferation of specific T cells (data not shown) suggesting that CTB-OVA was captured by DCs from the genital mucosa itself. Interestingly, the presenting capability of CD11c+ DCs is restricted to the CD11b+CD11c+ DC subset, whereas CD8+CD11c+ DCs as well as nonprofessional CD11c- APCs were unable to induce the proliferation of OVA-specific CD8 T cells (Fig. 4, C and D).

These results demonstrate that CTB increases the ability of DLN DCs to present OVA CD4 and CD8 epitopes derived from CTB-OVA acquired by genital route. The adjuvant property of CTB toward cellular responses against a colinked Ag may be at least partly explained by its carrier-delivery properties.

\[\text{FIGURE 3.} \text{ In vivo CTL activity after ivag immunization with OVA-conjugated CTB. Groups of wild-type C57BL/6 mice (n = 10) received three consecutive ivag immunizations at days 0, 14, and 21 with different formulations. OVA-specific cytolytic activity was measured in vivo following i.v. injection of equal numbers of CFSEhigh-labeled C57BL/6 splenocytes loaded with SIINFEKL peptide, and CFSElow-labeled non-pulsed C57BL/6 splenocytes used as targets into immunized mice at day 28. In vivo cytosis of these target cells was assessed ex vivo 15 h later by flow cytometry analysis of CFSEhigh or CFSElow cells in draining nodes (A), in spleen (B), and in vagina mucosa (C). Numbers above peaks represent the relative percentages of CFSEhigh or CFSElow cells. FACS profiles correspond to a representative experiment. D, Histograms represent the mean value (±SEM) of specific lysis percentages of four independent experiments. * Significant differences (p < 0.05, Bonferroni t test). ■, DLN; □, spleen; △, vagina mucosa.} \]
Cross-presentation of the CD8 epitope from the OVA-conjugated CTB by DC involves a proteasome-dependent and chloroquine-sensitive mechanism

We then evaluated the ability of DCs in contact with OVA-conjugated CTB to present OVA epitopes into the MHC class I-dependent pathway. This was determined by measuring the proliferation of CFSE-labeled TCR-OVA⁺ CD8⁺ T cells when incubated with BM-DCs preincubated with OVA-conjugated CTB in the presence or absence of inhibitors of both endocytic and class I degradation pathways. We chose to evaluate the inhibitory effect of the following drugs: lactacystin and epoxomicin known as potent irreversible proteasome inhibitors, brefeldin A described to inhibit the intracellular translocation of MHC class I-peptide complexes to the plasma membrane, and chloroquine which inhibits endosomal proteolysis.

Epoxomicin completely inhibited the presentation of the CD8 epitope (OVA257–264) derived from CTB-OVA at all inhibitor concentrations used (between 1 and 10 μM) as shown by the limited number of dividing cells (3%) (corresponding to 94% inhibition of specific proliferation) in comparison with the nontreated control (50% dividing cells). In contrast, the same drug partially inhibited proliferation following CTB-OVA in vitro uptake by BM-DCs in the presence of CT (Fig. 5). Lactacystin also induced a strong proliferation inhibition (82% inhibition corresponding to 9% of dividing cells for an inhibitor concentration of 20 μM) (data not shown). Moreover, brefeldin A at 10 μM partially blocked the proliferation of specific T cells (Fig. 5). Interestingly, the endosomal proteolysis inhibitor chloroquine strongly inhibited the presentation of the OVA257–264 Peptide by DCs incubated with OVA-conjugated CTB showing that the conjugate is internalized in endosomal vesicles and that this step is crucial for the presentation of MCH class I epitopes (Fig. 5). To note that the presentation of the exogenous peptide OVA257–264 (OVAp) by DCs, which does not require any processing, was not affected by all treatments.

These results demonstrate that DCs were able to take up the antigenic conjugate through a degradating mechanism involving endocytic compartments and the proteasome machinery and present the CD8 T cell epitope in the MHC I context. The adjuvant properties of CTB toward cytotoxic responses can be at least partly explained by its ability to deliver CD8 epitopes derived from colinked proteins in the MHC I compartment.

Discussion

This study demonstrates for the first time that ivag immunization with the CTB coupled to a soluble protein is able to induce regional MHC class I-dependent cytotoxic responses against the colinked Ag, as well as already described secretory and systemic Ab responses. Interestingly, CTB covalently linked to a protein Ag and administered by ivag route potentiates the ability of local DCs to present CD8 epitopes into the MHC class I-dependent pathway and to prime specific T cells. Thus, CTB adjuvant properties toward genital cytotoxic responses can be partly explained by the fact that CTB allows the in vivo uptake of the heterologous Ag in the mucosa by local DCs and favors the cross-presentation of CD8 epitopes.

The fact that OVA-conjugated CTB induces potent regional mixed Th and cytotoxic T cell responses against the colinked Ag when administered by genital route contrasts with the already well-described tolerogenic properties of CTB applied onto other mucosae. Indeed, CTB administered by oral or nasal route is described to suppress systemic cellular responses against colinked Ags (31, 32, 25). Our results are in accordance with data showing that ivag application of OVA protein during diestrus was unable to suppress subsequent DTH, whereas the same treatment applied during estrus induced peripheral tolerance, showing that the inductive immune properties of the reproductive tract are influenced by the menstrual cycle (33). More generally, unlike other mucosal sites such as the respiratory or gastrointestinal tracts, numerous works in humans and rodents demonstrate that the induction of immune responses and/or the susceptibility to infection in the reproductive tract are hormonally regulated (34). Thus, our data and other works suggest that progesterone treatment confers specific immune properties to the genital mucosa which may explain the adjuvant properties of CTB toward the effector responses observed here. The immunostimulatory properties of CTB toward Th1 responses following ivag immunization are not surprising as they were already demonstrated in a previous report from our laboratory showing that CTB applied onto skin, a nonmucosal pluristratified epithelium, is a strong adjuvant of Th1 responses against coadministered Ags (35).

The present data demonstrate that CTB applied ivag with CT potentiates mixed T cell responses, whereas it is described to favor Th2 responses following intranasal immunization, supporting the fact that the genital mucosa has specific properties different from other mucosae (Table I and Ref. 36).

Furthermore, the observation that the covalent coupling of the Ag to CTB is necessary to induce CTL responses in vivo by genital route suggests that CTB favors the delivery of the Ag into the MHC class I pathway. These results are in accordance with another study demonstrating that the nontoxic B subunit of Shiga toxin administered by systemic route is an adjuvant of in vivo CTL responses only when fused to a tumor Ag (20). In the same sense, the inability of the nontoxic B subunit of LT coadministered with OVA by mucosal route to induce CTL responses support this conclusion (11). On the contrary, mucosal immunization with CT evokes strong CTL responses against a coadministered Ag independently of their covalent coupling as described here and in previous works (Fig. 3 and data not shown, Refs. 9, 10, and 12). In the same way, LT mutants are potent mucosal adjuvants of CTL responses against coadministered Ags without any need of coupling (11). Altogether, our results and published data strongly suggest that the mucosal adjuvant properties of CT and CTB toward mucosal CTL responses involve distinct mechanisms to elucidate.

This work confirms previous data demonstrating that CTB devoid of toxic activity, administered by ivag route, is an adjuvant of...
systemic and genital Ab responses against itself and a coadministered heterologous Ag (Ref. 13 and third section of Results). The present study further indicates that the covalent coupling of the Ag to the CTB molecule is necessary to induce Ab responses against the coadministered Ag. In accordance with numerous other studies, the addition of native CT, one of the most potent mucosal adjuvant, potentiates these responses (13, 37, 38). The low titters in specific Abs present in vaginal secretions may be explained by the fact that the prolonged diestrus stage induced following Deproprovera treatment is described to diminish the presence of IgG and IgA in vaginal washes (39). Experiments using other progestative molecules are underway to verify this hypothesis in our model.

Following ivag immunization, CTB allows the delivery and presentation of immunodominant CD4 and CD8 OVA epitopes by CD11c− DCs from DLN but not by CD11c+ cells (Fig. 4). These results are in accordance with previous data by Zhao et al. (16) demonstrating that CD11c+ DLN DCs were in charge to present CD4 epitopes derived from HSV-2 following genital infection. The observation that CD4 and CD8 OVA epitopes are presented by CD11c− DLN DCs 36 h after immunization suggests that genital DCs take up the Ag at the site of immunization and then migrate to DLN to prime specific T cells. The fact that CD11c− DCs, purified from DLN at a shorter time point following ivag immunization (data not shown), are unable to activate specific T cells support this hypothesis. This excludes the possibility that the protein Ag migrates alone through the circulation to be presented by resident lymph node DCs as previously shown following s.c. immunization (40). At least two subsets of DCs present in the genital mucosa, one in the pluristratified epithelium (Langerhans-like DCs) and the other one in the lamina propria (submucosal DCs) (C. Luci, unpublished data and Ref. 16), might be involved in the uptake of the Ags covalently linked to CTB in the mucosa itself. In that sense, the observation that the DLN CD11b+ DC subset but not the CD8+ DC subset is able to present a MHC class I epitope suggests, as previously observed by Zhao et al. (16), a MHC class II epitope that the genital Ag is captured by submucosal DCs. A possible explanation is that these DCs pick up the Ag directly from the mucosal lumen as previously proposed (41). Alternatively, DCs in the mucosa might take up the Ag from epithelial cells as the mucosal lumen as previously proposed (41). Alternatively, DCs in the mucosa might take up the Ag from epithelial cells as previously proposed (41). Alternatively, DCs in the mucosa might take up the Ag from epithelial cells as previously proposed (41). Alternatively, DCs in the mucosa might take up the Ag from epithelial cells as previously proposed (41).

Our results showing that CD11c− DLN DCs from mice immunized with OVA alone are not efficient to activate specific CD4 and CD8 T support the notion that CTB improves the uptake of the Ag by DCs and its processing into the MHC class I and II pathways. Our study confirms previous in vitro data showing that the covalent coupling of Ags to CTB increases the ability of BM-DCs to activate Ag-specific CD4 T cells (43) and CD8 T cells (22). In vitro inhibition studies with classical drugs blocking crucial steps of MHC class I and class II pathways led us to further understand the mechanism by which CTB allows the cross-presentation of CD8 epitopes derived from exogenous OVA-conjugated CTB. The complete abolition of presentation of the OVA-derived CD8 epitope by specific proteasome inhibitors (epoxomicin, lactacycin) and the partial inhibition by brefeldin A demonstrate that CTB delivers the exogenous protein into the cytosolic conventional MHC class I pathway (Fig. 5). This result corroborates previous work in which the nontoxic Shiga toxin B subunit elicits CTL responses against a fused Ag by a proteasome-dependent mechanism (20). Furthermore, the observation that an inhibitor of receptor-mediated endocytosis (chloroquine) significantly affects MHC class I presentation suggests that at least a part of the CTB− OVA conjugate is internalized in acidic vesicles from which a fraction of Ag gains access to the retrograde transport pathway for MHC class I presentation. This explanation is supported by previous in vitro data showing that endocytosis of CTB in pig and human cell lines is mediated in part by a clathrin-dependent mechanism leading to the targeting of Ags into endosomes (44, 45).

In conclusion, CTB administered by ivag route can serve as an effective adjuvant and Ag-delivery system toward mucosal Ab and cytotoxic responses for nonreplicating Ags, suggesting that this type of formulation might be adequate to elicit protective immune responses limiting the entry of infectious pathogens at genital mucosa.

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Disclosures

The authors have no financial conflict of interest.

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